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Causes of Papillomas on Fish Living in Chlorinated Sewage Effluent

Auburn Univ., AL

Prepared for

Environmental Research Lab.
Gulf Breeze, FL

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October 1982

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CAUSES OF PAPILLOMAS ON FISH LIVING IN CHLORINATED SEWAGE EFFLUENT

by

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CR807844010

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OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
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16. ABSTRACT

This research was initiated to determine the cause of the oral papillomas on black bullheads (Ictalurus melas) from the final oxidation pond of the Tuskegee, Alabama, sewage treatment plant. Ames-test mutagenicity of a pond-water concentrate indicated the presence of a chemical carcinogen in the pond water. However, water and sediment analysis did not identify substances suspected of causing the tumors. Cytoplasmic inclusion bodies were present in some papilloma cells, but attempts to find virions in the tumor using electron microscopy and to transmit the papillomas by injection of cell-free tumor homogenate into black bullheads were not successful. Juvenile black bullheads, yellow bullheads (Ictalurus natalis), and channel catfish (Ictalurus punctatus) were confined to cages in the oxidation pond and in a control pond. Caged fish did not develop neoplasms but most caged black bullheads in the oxidation pond developed focal, oral hyperplasia and stomatitis in the same mouth locations occupied by papillomas in wild black bullheads from this pond.

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FOREWORD

The protection of our estuarine and coastal areas from damage caused by toxic organic pollutants requires that regulations restricting the introduction of these compounds into the environment be formulated on a sound scientific basis. Accurate information describing dose-response relationships for organisms and ecosystems under varying conditions is required. The EPA Environmental Research Laboratory, Gulf Breeze, contributes to this information through research programs aimed at determining:

the effects of toxic organic pollutants on individual species and communities of organisms;

the effects of toxic organics on ecosystem processes and components;

the significance of chemical carcinogens in the estuarine and marine environments.

Few studies of carcinogenic effects in field populations of fishes have been conducted. This report describes results from a unique study of a population of catfish with an unusually high prevalence of tumors in a finite body of water, an effluent pond of a sewage treatment plant. Results indicated that substances isolated from pond waters were mutagenic, and suggested the possibility of the presence of chemical carcinogens. These data may provide early evidence of a link between contaminated water and tumor prevalence in a fish population.

Henry F. Enos
Director
Environmental Research Laboratory
Gulf Breeze, Florida

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ABSTRACT

This research was initiated to determine the cause of oral papillomas in black bullheads (Ictalurus melas) from the final oxidation pond of the Tuskegee, Alabama, sewage treatment plant. The water in this pond was chlorinated effluent from the sewage treatment plant. Ames-test mutagenicity of a pond-water concentrate indicated the presence of a chemical carcinogen in the pond water. However, water and sediment analysis did not identify substances suspected of causing the tumors. Cytoplasmic inclusion bodies were present in some papilloma cells, but attempts to find virions in the tumor using electron microscopy and to transmit the papillomas by injection of cell-free tumor homogenate into black bullheads were not successful. Juvenile black bullheads, yellow bullheads (Ictalurus natalis), and channel catfish (Ictalurus punctatus) were confined to cages in the oxidation pond and in a control pond. Most caged black bullheads in the oxidation pond developed focal, oral hyperplasia and stomatitis in the same mouth locations occupied by papillomas in wild black bullheads from this pond. These hyperplastic lesions healed in most fish during additional exposure. Mucosal hyperplasia also occurred, but at a much lower incidence, in caged yellow bullheads and channel catfish and in controls of all species. A similar incidence of hyperplastic lesions in black bullheads in floating and sunken cages indicated that contact with the sediment or ingestion of benthic food organisms did not affect pathogenesis of the lesions.

This report was submitted in fulfillment of Grant No. CR807844010 by Auburn University under the sponsorship of the U.S. Environmental Protection Agency. This report covers the period from 1 October 1980, to 30 September 1981, and work was completed as of 30 September 1981.

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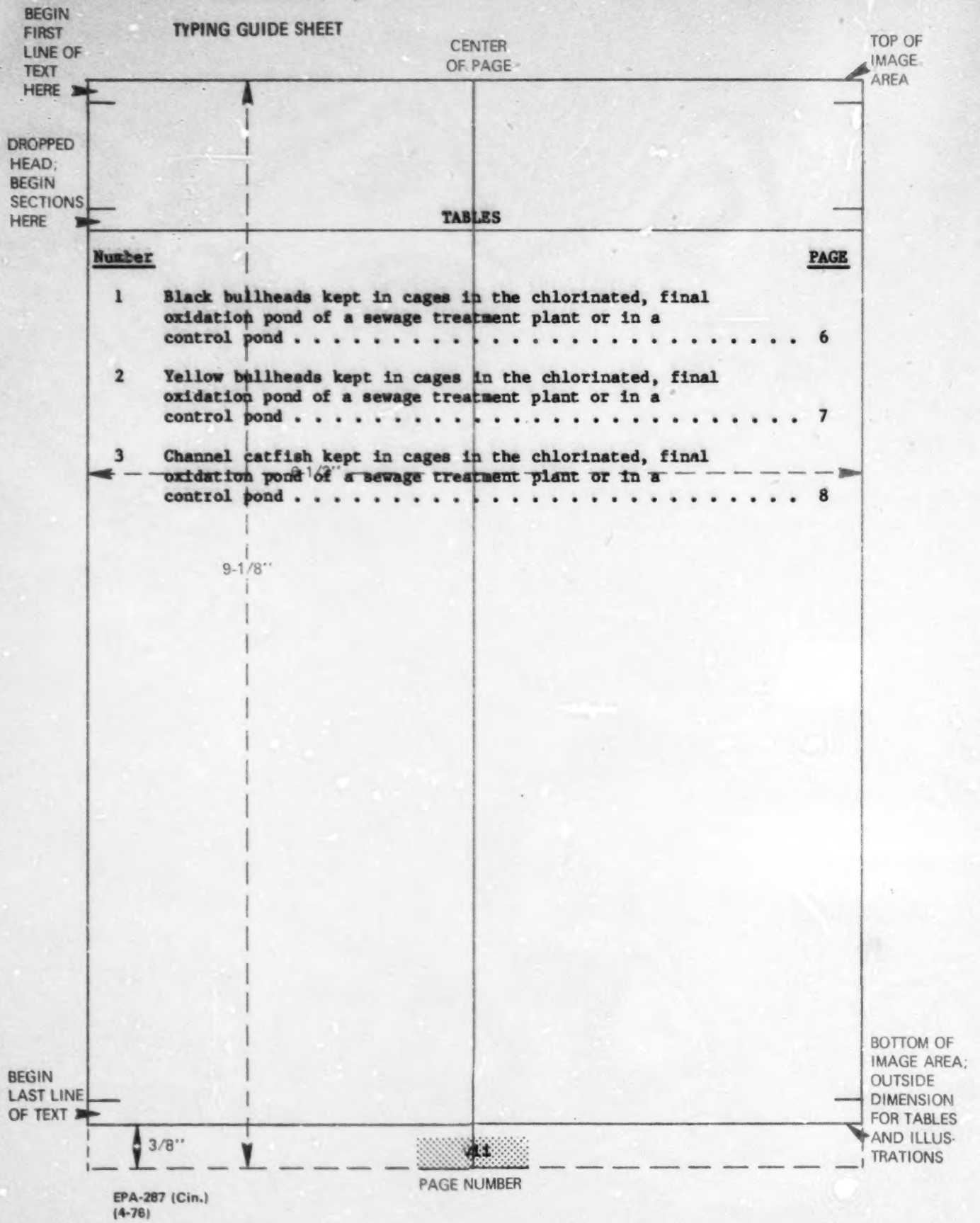
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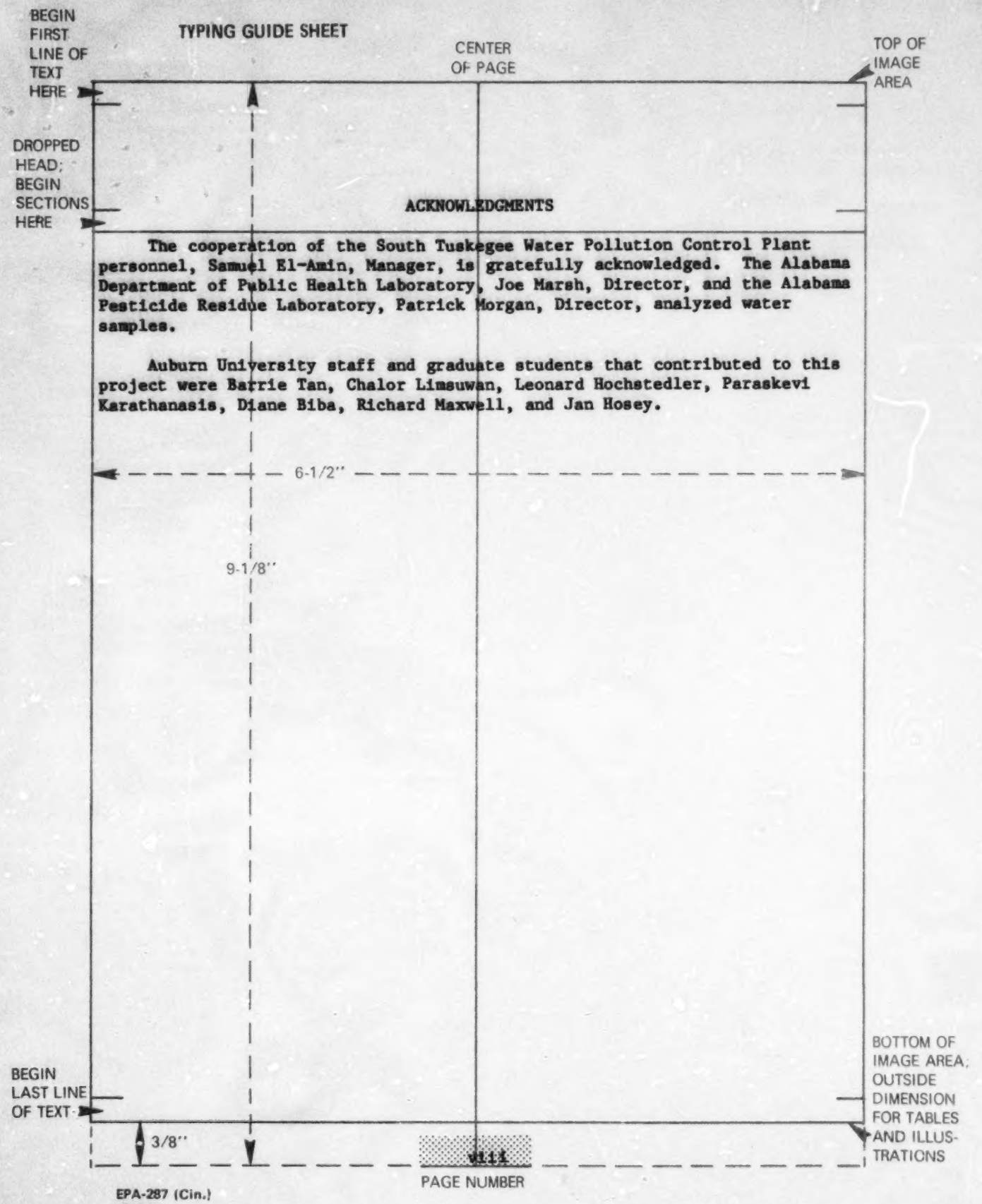
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The cooperation of the South Tuskegee Water Pollution Control Plant personnel, Samuel El-Amin, Manager, is gratefully acknowledged. The Alabama Department of Public Health Laboratory, Joe Marsh, Director, and the Alabama Pesticide Residue Laboratory, Patrick Morgan, Director, analyzed water samples.
Auburn University staff and graduate students that contributed to this project were Barrie Tan, Chalor Limsuwan, Leonard Hochstedler, Paraskevi Karathanasis, Diane Biba, Richard Maxwell, and Jan Hosey.
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SECTION 1

INTRODUCTION

A population of black bullheads in the final oxidation pond of the City of Tuskegee, Alabama, sewage treatment plant had a 70% prevalence of oral papillomas (Grizzle et al. 1981). These fish provided an opportunity to study the etiology of fish papillomas, especially as related to chlorinated sewage effluent.

Sewage entering this treatment plant did not include discharge from industries or institutions, but run-off water from residential and agricultural areas entered the plant after rains. The process of chlorinating the sewage after it passed through an aeration basin and a settling basin was considered a possible source of carcinogens.

There has been only one previous report of a high prevalence of neoplasms in aquatic animals living in chlorinated sewage effluent (Rose 1977). In that case, a pond at Reese Air Force Base, Texas, contained tiger salamanders (Ambystoma tigrinum) that had neoplastic and non-neoplastic lesions with a peak frequency of 53%. Chemical carcinogens, especially perylene, were suspected of causing the lesions.

Papillomas occur on many species of fish and have been associated with pollution (Lucke and Schlumberger 1941; Russell and Kotin 1957; Cooper and Keller 1969; Sonstegard 1977; Dawe and Harshbarger 1975) and tumorigenic viruses (Edwards et al. 1977; Schwanz-Pfitzner 1976; McAllister et al. 1977). Other fish tumors have a genetic origin (Siciliano et al. 1971; Vielkind et al. 1971). All of these factors were considered as possible causes of the papillomas in fish from the Tuskegee sewage treatment plant.

The results obtained during this grant period are preliminary because of the nature of this project and are under continued study. Objectives of this study were as follows:

1. determine if tumors will develop in healthy fish (Ictalurus spp.) placed in the sewage pond and the time required for tumor development.
2. describe the histology and ultrastructure of tumors on fish from the sewage pond.
3. analyze water from the sewage pond for toxic or carcinogenic chemicals.
4. determine if mutagenic substances are present in water from the sewage pond.
5. attempt transmission of oxidation-pond fish papillomas to healthy black bullheads by injecting cell-free tumor homogenates.

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SECTION 2

CONCLUSIONS AND RECOMMENDATIONS

The Tuskegee, Alabama, sewage treatment plant final oxidation pond had wild black bullheads with a 70% prevalence of oral papillomas. Results of this study indicated that the acidic organic solvent extract of this oxidation pond water was mutagenic to Salmonella typhimurium bacterial tester strains TA98 and TA100 if activated with Aroclor-induced rat liver enzymes. No evidence of a viral etiology for the papillomas was found during ultrastructural studies of the lesions or during transmission attempts.

Water analyses revealed no chemicals in sufficient concentration to cause the papillomas. The possibility of temporal changes in composition of the water was not adequately considered during this study. Future investigations should include collection of water samples during different times of the year.

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Caged black bullheads in the oxidation pond developed focal epithelial hyperplasia and stomatitis in the same mouth location occupied by papillomas in wild black bullheads. The lesions in caged fish may be related to chemicals in the water because no pathogens were consistently associated with these lesions. Neoplasms did not develop in caged fish kept in the oxidation pond. Whether additional exposure time will lead to development of tumors can only be determined by longer exposure.

The conclusions of this study are based on preliminary findings. All phases of this study will continue and new information obtained from additional research could modify the conclusions of this report.

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SECTION 3

STUDY SITE

The South Tuskegee Water Pollution Control Plant is located 7 km southwest of Tuskegee, Alabama. The plant began operation during January 1977 and has had an average daily flow of 4×10^6 liters/day. The sewage treatment process is illustrated in Figure 1. The chlorine residual leaving the contact chamber has varied from 0 to 4 mg/l.

The surface area of the final oxidation pond is 1.0 hectare with a maximum depth of 3 meters. Four mechanical aerators in the final oxidation pond prevent stratification and maintain dissolved oxygen near 100% saturation (Figure 2). The pond has an earthen bottom with a concrete apron at the water-surface line. A dike surrounding the pond prevents direct runoff drainage from entering it. At a flow rate of 4×10^6 liters/day, water retention time in the pond is approximately 5 days.

The portion of Tuskegee's sewage that entered this plant contained only domestic wastes; however, following heavy rainfall the flow rate into the plant increased greatly indicating that surface runoff from surrounding agricultural and residential areas entered the sewage system.

When the first collections from the oxidation pond were made during November 1979, the fish species present were black bullheads, green sunfish (Lepomis cyanellus), and golden shiners (Notemigonus crysoleucas). The origin of these fishes was not determined, but these species are common in nearby ponds. A total of 2,000 silver carp (Hypophthalmichthys molitrix) was released into the oxidation pond during May and July 1980 to control algal density.

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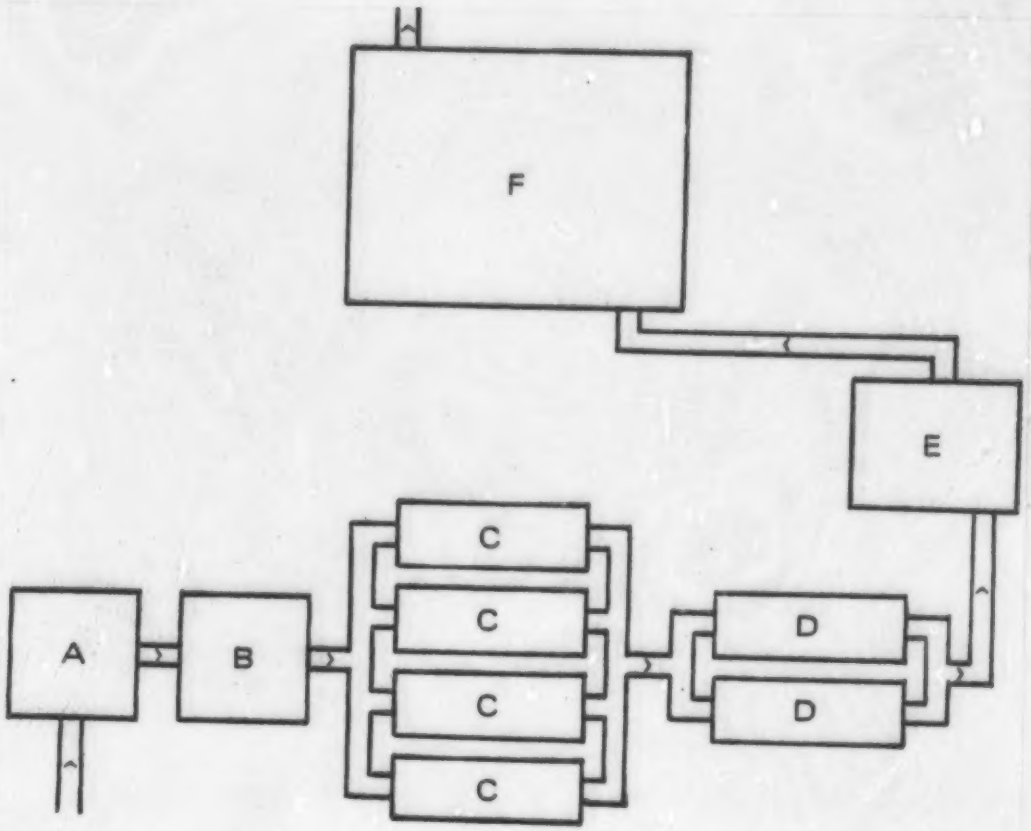

Figure 1. Schematic plan of the South Tuskegee Water Pollution Control Plant. A, pumping station; B, grit removal; C, aeration basins; D, settling basins; E, chlorine contact chamber; F, final oxidation pond.

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SECTION 4

MATERIALS AND METHODS

EXPOSURE OF FISH TO WATER IN THE FINAL OXIDATION POND

Three species of fishes were placed in cages: black bullheads (125 mm average total length) obtained by draining an unmanaged farm pond located 4 km southwest of Tuskegee, Alabama; yellow bullheads (80 mm average total length) from a fish farm near Clio, Alabama; and channel catfish (130 mm average total length) produced at the Fisheries Unit of the Auburn University Agricultural Experiment Station. All of these fish hatched during May or June 1980. The cylindrical cages were constructed of plastic netting supported by steel hoops. Black bullheads were placed in 1.1-m^3 cages of 19-mm mesh netting; other species were placed in 0.7-m^3 cages of 6-mm mesh netting. Cages were allowed to rest on the pond bottom (sinking cages) or were suspended off the bottom by floats (floating cages).

Cages were placed at three locations in the oxidation pond of the sewage treatment plant (Figure 2): near the inlet (inlet A), 75 meters from the inlet (inlet B), and near the outlet. Caged control fish were placed in a 0.04- hectare earthen pond at the Auburn University Agricultural Experiment Station. Stocking dates are indicated in Tables 1, 2, and 3. All fish were fed a commercial catfish ration (45% protein). Fish were fed only twice a week (approximately 3% of body weight at each feeding) to encourage utilization of natural food.

Chemical treatments for infectious diseases were not used immediately before or during the experiment; however, the channel catfish and yellow bullheads may have been exposed to formalin, potassium permanganate, or other chemicals routinely used in commercial fish culture. Pathogens, especially the protozoan, *Ichthyophthirius multifilis*, and the bacterium, *Flexibacter columnaris*, killed many of the caged fish. Infectious diseases killed all of the black bullheads soon after stocking in the control pond on 21 October 1980. The first cages were stocked with 100 fish per cage, but to reduce deaths caused by infectious diseases, other cages were stocked with 50 fish per cage.

Moribund fish from the cages were examined for pathogens. Liver and trunk kidney tissues were streaked on brain-heart infusion agar, and fresh gill and skin scrapings were examined with a compound microscope. Other organs were examined grossly for presence of parasites or lesions.

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Table 1. Black bullheads kept in cages in the chlorinated, final oxidation pond of a sewage treatment plant or in a control pond.

Cage Location	Date Examined	Cage Type	Length of Exposure (Days)	Number of Fish Examined	Percent with lesion[a]
Inlet A	21 Oct 80	Sinking	0	100	0
Inlet A	21 Oct 80	Floating	0	100	0
Inlet A	12 Dec 80	Floating	52	3	33
Inlet B	23 Feb 81	Sinking	0	50	0
Inlet B	23 Feb 81	Floating	0	50	0
Inlet B	11 Mar 81	Sinking	16	28	4
Inlet B	11 Mar 81	Floating	16	21	5
Inlet B	7 Apr 81	Sinking	43	12	50
Inlet B	7 Apr 81	Floating	43	3	0
Inlet B	11 Jun 81	Sinking	109	9	0
Inlet B	4 Aug 81	Sinking	164	6	33
Outlet	21 Oct 80	Sinking	0	100	0
Outlet	21 Oct 80	Floating	0	100	0
Outlet	11 Mar 80	Sinking	141	25	80
Outlet	11 Mar 81	Floating	141	33	79
Outlet	7 Apr 81	Sinking	168	22	95
Outlet	7 Apr 81	Floating	168	33	88
Outlet	11 Jun 81	Sinking	234	21	9.5
Outlet	11 Jun 81	Floating	234	28	7
Outlet	4 Aug 81	Sinking	289	11	9
Outlet	4 Aug 81	Floating	289	7	0
Control	24 Nov 80	Sinking	0	50	0
Control	24 Nov 80	Floating	0	50	0
Control	13 Mar 81	Sinking	109	24	4
Control	13 Mar 81	Floating	109	31	3
Control	5 May 81	Sinking	162	23	0
Control	5 May 81	Floating	162	30	0
Control	17 Jun 81	Sinking	205	5	0
Control	17 Jun 81	Floating	205	25	0
Control	6 Aug 81	Sinking	256	3	0
Control	6 Aug 81	Floating	256	8	0

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[a]Grossly visible hyperplasia of the oral mucosa.

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Table 2. Yellow bullheads kept in cages in the chlorinated, final oxidation pond of a sewage treatment plant or in a control pond.

Cage Location	Date Examined	Cage Type	Length of Exposure (Days)	Number of Fish Examined	Percent with lesion[a]
Inlet B	12 May 81	Floating	0	50	0
Inlet B	4 Aug 81	Floating	85	4	0
Outlet	12 May 81	Floating	0	50	0
Outlet	11 Jun 81	Floating	30	10	0
Outlet	4 Aug 81	Floating	85	8	0
Control	12 May 81	Floating	0	50	0
Control	17 Jun 81	Floating	36	7	14
Control	6 Aug 81	Floating	87	3	0

6-1/2"

[a]Grossly visible hyperplasia of the oral mucosa.

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Table 3. Channel catfish kept in cages in the chlorinated, final oxidation pond of a sewage treatment plant or in a control pond.

Cage Location	Date Examined	Cage Type	Length of Exposure (Days)	Number of Fish Examined	Percent with lesion[a]
Inlet A	16 Jan 81	Sinking	0	50	0
Inlet A[b]	16 Jan 81	Floating	0	50	0
Inlet B	28 Jan 81	Sinking	12	22	0
Inlet B	11 Mar 81	Sinking	54	22	0
Inlet B	11 Jun 81	Sinking	146	12	0
Inlet B	4 Aug 81	Sinking	200	10	0
Outlet	28 Jan 81	Sinking	0	50	0
Outlet	28 Jan 81	Floating	0	50	0
Outlet	11 Mar 81	Floating	42	47	0
Outlet	7 Apr 81	Sinking	69	49	0
Outlet	11 Jun 81	Sinking	122	29	3.4
Outlet	11 Jun 81	Floating	122	18	5.5
Outlet	4 Aug 81	Sinking	177	26	0
Outlet	4 Aug 81	Floating	177	18	5.5
Control	10 Feb 81	Sinking	0	50	0
Control	10 Feb 81	Floating	0	50	0
Control	17 Jun 81	Sinking	131	43	2.3
Control	17 Jun 81	Floating	131	46	0
Control	6 Aug 81	Sinking	182	24	8.3
Control	6 Aug 81	Floating	182	43	0

[a] Grossly visible hyperplasia of the oral mucosa.

[b] Cage moved from inlet A location to inlet B.

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PERSISTENCE OF PAPILLOMAS

Adult black bullheads with oral papillomas were captured from the oxidation pond with gill nets or traps on 22 April 1980 and 31 March 1981. The maximum length and width and approximate thickness of the tumors were recorded, then the fish were tagged and released into a 3-m diameter plastic pool at the Auburn University Agricultural Experiment Station, 5 fish per pool. Of the 28 fish tagged, only 6 lived for more than a month because of infectious diseases. One fish was examined after 57 days, and the other five were examined after 124 days. One of the fish lived for 378 days and was sacrificed for histological examination.

HISTOLOGY

Lesions, oral mucosa, gills, and livers of wild fish from the oxidation pond and from the cage experiment were fixed in Bouin's, Zenker's, or neutral buffered 10% formalin fixative. Tissues were dehydrated in alcohol, infiltrated with paraffin, sectioned (7 um), and stained with Harris' hematoxylin and eosin. Masson's trichrome stain and the Feulgen and PAS reactions were used on selected sections.

9-1/8"

ELECTRON MICROSCOPY

Lesions and oral mucosa were fixed in 3% phosphate-buffered glutaraldehyde and post-fixed in 1% osmium tetroxide. Tissues were dehydrated in ethyl alcohol and infiltrated and embedded in epoxy. Thick (1 um) and thin sections (60-90 nm) were cut with glass knives. Thick sections were placed on glass slides, stained with toluidine blue, and used for orientation. Thin sections were mounted on copper grids and stained with uranyl acetate and lead citrate.

WATER ANALYSIS

Metal concentrations were determined by the Soil Testing Laboratory, Auburn University, using atomic absorption spectrophotometry. These water samples were acidified with nitric acid immediately after collection. The Alabama Pesticide Residue Laboratory, Auburn, Alabama, used gas chromatography to scan water samples for pesticides. The Alabama Department of Public Health Laboratory, Montgomery, Alabama, also used gas chromatography to detect organic compounds in water. Other water analyses were available from the Tuskegee Water Pollution Control Plant.

MUTAGENICITY TESTS

Concentrates of oxidation-pond water, collected at the pond outlet, were tested for mutagenicity with the Ames test (Ames et al. 1975). Each petri plate contained 20 ml of base agar (2.7% minimum agar Davis; Difco) covered

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with 3.2 ml of top agar that included the S-9 mix (if required), the bacterial tester strain, and the substance being tested. The S-9 mix consisted of 0.1 ml of Aroclor-induced rat liver enzymes (S-9; Sigma). The positive control mutagen, benzo(a)pyrene (B(a)P), and water concentrates were dissolved in dimethylsulfoxide (DMSO; filter sterilized) and tested with and without the S-9 mix. Controls contained DMSO, S-9 mix, or hexane residue (prepared by distilling 16 liters of hexane) in concentrations corresponding to those in test petri plates. B(a)P concentrations ranged from 1.0 to 25 ug per plate, and dry residues of the pond water were tested in concentrations from 0.3 to 3 mg per plate. Five replications were used for most tests. An acidic fraction from 2 liters of oxidation-pond water was obtained by adjusting the pH to 1.0 with HCl and extracting three times with 200 ml of 25% ether and 75% hexane. A separatory funnel was used to separate the water from the solvents. The extracted solution was dried with dry air at 30-40 C. The yield was 1.7 mg of dry residue per liter of water. A basic fraction of the oxidation pond water was obtained by adjusting the 2-liter water sample pH to 12-13 with NaOH and extracting three times with 25% ether and 75% hexane. This extract was separated and dried by the same method used for the acidic fraction. The yield was 0.9-1.0 mg of dry residue per liter of water. The basic fraction required the addition of formic acid before it could be dissolved in DMSO. The acidic and basic fractions were tested with Salmonella typhimurium strains TA98 and TA100. The water residues were filter sterilized before addition to the agar.

TUMOR TRANSMISSION

During January 1979, a papilloma from an oxidation-pond black bullhead was homogenized in Hank's balanced salt solution (HBSS), passed through a 0.45-um filter and injected subcutaneously into the mouth fornices of 3 adult black bullheads. Control black bullheads were injected with filtered HBSS. Each fish was kept in a separate 40-liter glass aquarium, fed a commercial catfish ration, and examined at approximately monthly intervals.

Four papillomas were homogenized in HBSS, filtered through a 0.45-um membrane, and lyophilized in separate ampules to concentrate the homogenate. One-year-old black bullheads were acclimated to 40-liter glass aquaria, one fish per aquarium. During May 1981, the lyophilized papilloma homogenates were injected into 16 fish after suspending it in distilled water one-half of the original volume. Five controls were injected with filtered HBSS. All fish were injected subcutaneously at the right mouth fornix and also intraperitoneally. Fish were fed daily and examined monthly.

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SECTION 5

RESULTS

EXPOSURE OF FISH TO WATER IN THE FINAL OXIDATION POND

Of the 200 black bullheads stocked in 2 cages near the inlet of the oxidation pond (inlet A; Figure 2), only 3 were alive after 52 days (Table 1). One of these survivors had grossly visible, focal hyperplasia of the oral mucosa. Similar lesions developed later on other caged black bullheads (Figure 3). The highest frequency of this lesion occurred after 168 days in black bullheads at the pond outlet location (Table 1). The incidence of oral mucosa hyperplasia in black bullheads at the oxidation pond outlet and inlet locations cannot be accurately compared because of low survival in cages at the inlet locations, but 50% of the black bullheads in the sinking cage at inlet B had oral mucosa hyperplasia after only 43 days.

The grossly visible hyperplasia did not persist. These lesions had regressed on fish in cages at inlet B and outlet locations after 109 days and 234 days, respectively, and the regression occurred between 7 April 1981 and 11 June 1981 in both locations.

Caged yellow bullheads (Table 2) in oxidation and control ponds experienced high mortality during the first month after stocking. Survival was higher, in all locations for the caged channel catfish (Table 3) than for the black bullheads and yellow bullheads. Oral lesions similar to those in caged black bullheads occurred in channel catfish at the outlet, but the incidence of these lesions was similar in control channel catfish. Focal, oral mucosa hyperplasia in control fish of all species ranged from 0 to 14%.

PERSISTENCE OF PAPILLOMAS

Size of the two, oral papillomas did not change in the one black bullhead examined 57 days after removal from the oxidation pond. Four of the 8 papillomas in the 5 fish examined after 124 days were approximately one-half the original area, 2 decreased slightly in one fish, one disappeared, and one increased slightly. One fish that had a papilloma reduced to one-half size after 124 days retained the papilloma at this new size until necropsied after 378 days.

HISTOLOGY

The papillomas on wild black bullheads from the oxidation pond consisted of a hyperplastic mucosa covering papillae of submucosa (Figures 4 and 5). Goblet cells and alarm substance cells were absent from parts of the mucosal

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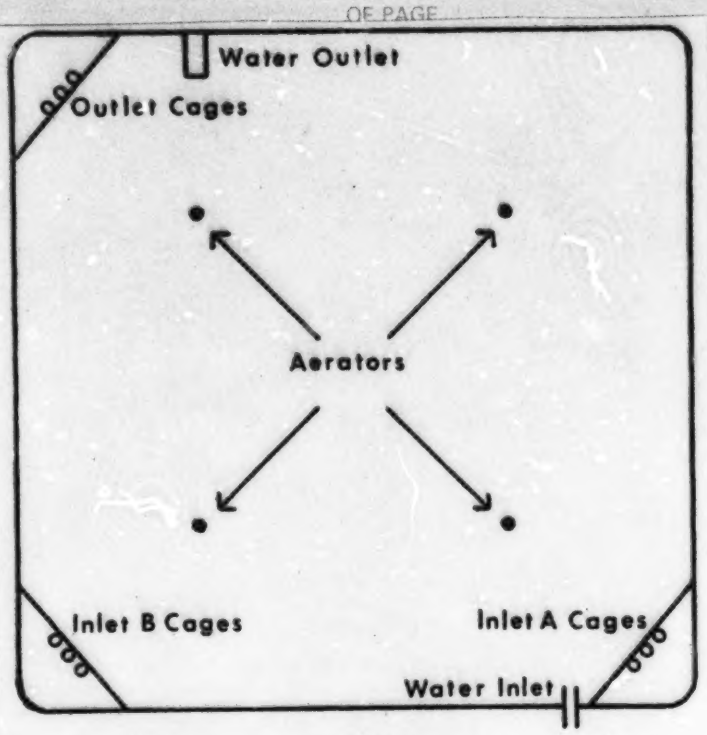

Figure 2. Fish cage locations in the final oxidation pond of the South Tuskegee Water Pollution Control Plant.

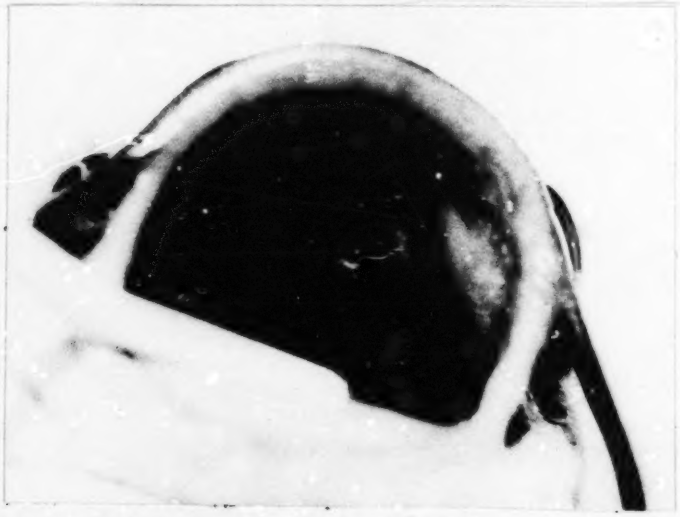

Figure 3. Oral lesion in a juvenile black bullhead from a sinking cage near the outlet of the final oxidation pond. This is a lesion typical of those seen in caged fish during this study.

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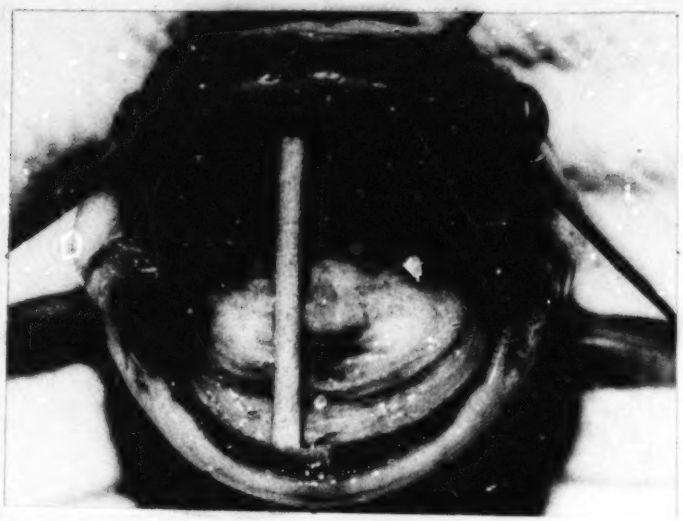

Figure 4. Oral papillomas in a wild black bullhead from the final oxidation pond.

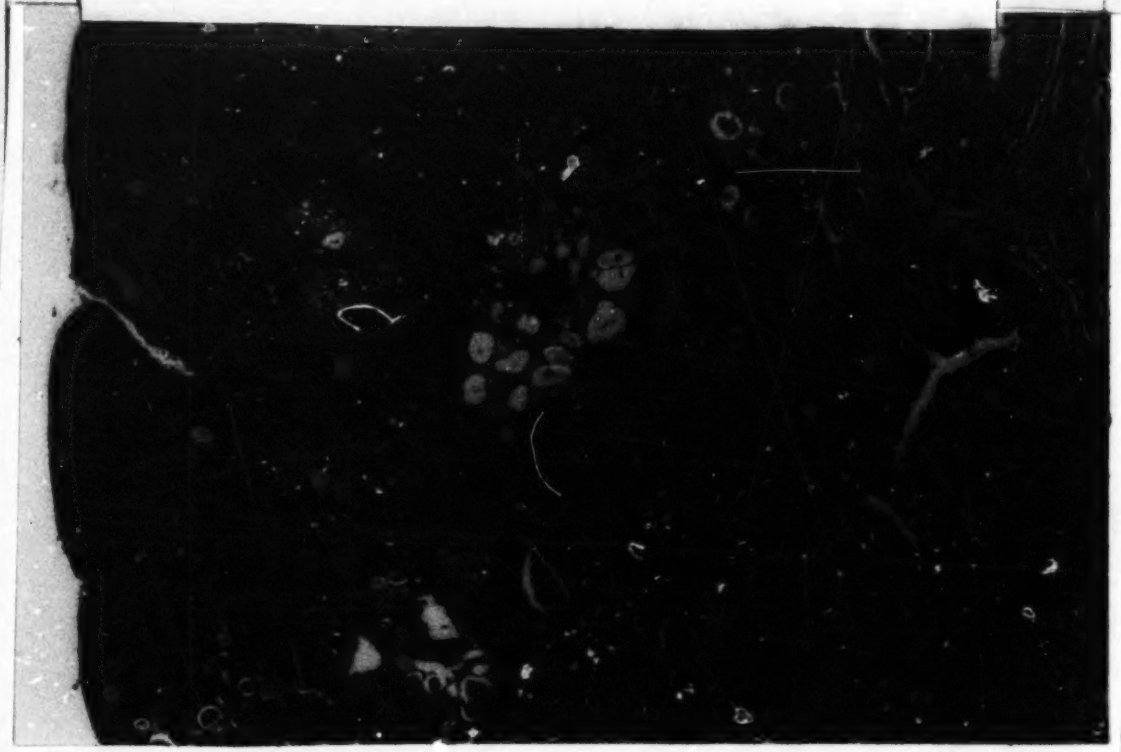

Figure 5. Paraffin section of an oral papilloma from a final oxidation pond black bullhead (X65, hematoxylin and eosin).

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portion. Malpighian cells near the surface were usually normal in shape but the mid to basal portion of the mucosa consisted of stellate cells. Cytoplasmic inclusion bodies, usually in cells near the surface, were eosinophilic, PAS positive, and weakly Feulgen positive. The number of mitotic figures was similar to that of normal tissue. The tumors were not invasive and there were no metastasis. Papillomas near the lip were often black and contained numerous melanocytes. Necrosis and stomatitis occurred in association with some papillomas, usually those having large surface areas. Approximately 200 adult black bullheads were examined from the final oxidation pond; 70% had neoplasms. These lesions were all papillomas located in or near the mouth fornices, except for one papilloma on the dorsal head, one dermal fibroma (Figure 6) on the dorsal head, and three papillomas in other areas of the oral cavity or pharynx.

The grossly visible hyperplasia in caged fish in the oxidation and control ponds was located in the mouth fornix, except for two fish with similar lesions of the oral valve near the middle of the mouth. Most of these lesions (Figure 7) consisted of a hyperplastic mucosa approximately 600 um thick, compared to a normal thickness of 20-100 um. The underlying submucosa was more vascular and up to ten times thicker than normal. Stomatitis, indicated by congestion and infiltration by lymphocytes, was present in the submucosa of most lesions. The hyperplastic epithelium consisted of stellate cells separated by non-staining areas, possibly indicating spongiosis (Figure 8). Goblet cells and alarm substance cells were less common than in normal oral mucosa.

ULTRASTRUCTURE

In early studies, electron-dense cells with spherical zones having less dense central areas containing 35-nm particles were found in the wild black bullhead papillomas (Grizzle et al. 1981). These cells were not found in recently examined papillomas, perhaps because most sections were cut through the spongy zone of the papillomas.

The stellate cells of the mucosal portion of the papilloma (Figure 9) were separated by intercellular spaces except for small zones where cells were held together by desmosomes (Figure 10). The space between cells contained amorphous material and particles resembling glycogen.

Sections through the papilloma inclusion bodies have not been made. Those peripheral areas of the papillomas examined consisted of epithelial cells resembling normal oral mucosa epithelium.

WATER ANALYSIS

Between September 1980 and August 1981, water leaving the final oxidation pond had 0.3 to 7.0 mg/l ammonia-nitrogen (average 1.6 mg/l) and a pH between 5.2 and 7.0. During this period, total residual chlorine of water leaving the contact chamber was 0.2 to 1.0 mg/l (average 0.4 mg/l) and was lower than during 1979 when the black bullhead papillomas were first discovered (Figure 11).

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Figure 6. Dermal fibroma from the dorsal head of a wild black bullhead collected from the final oxidation pond (X130, hematoxylin and eosin).

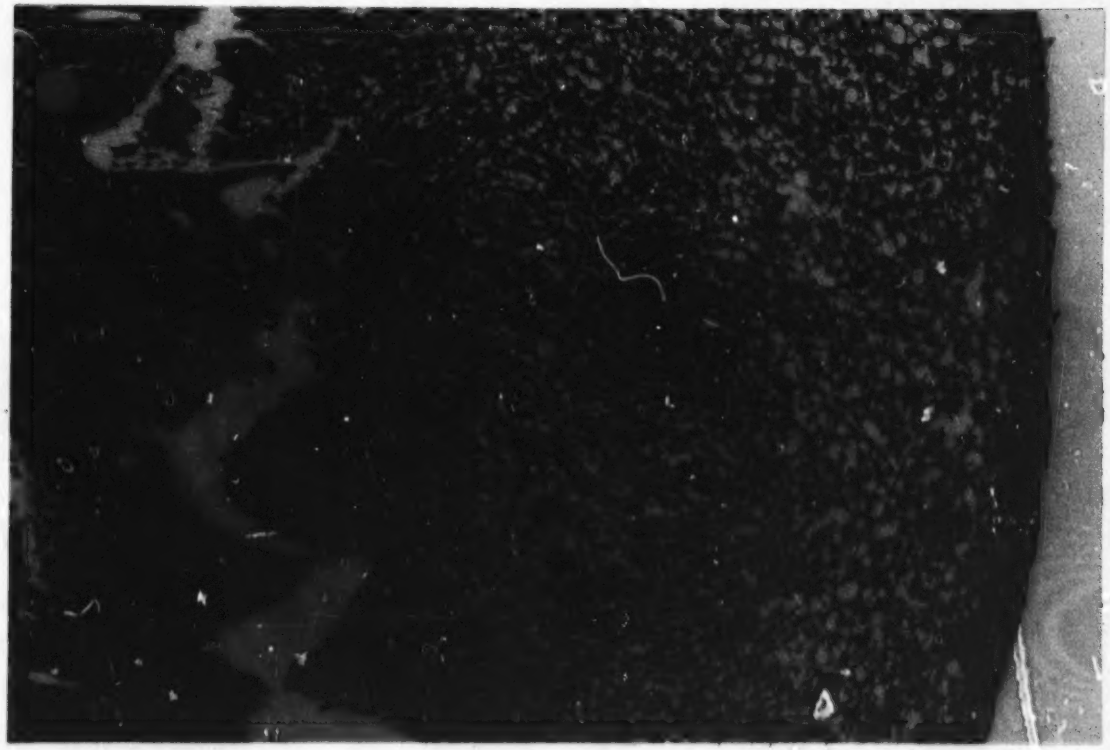

Figure 7. Oral lesion from a juvenile black bullhead confined in a cage in the final oxidation pond for 168 days. The hyperplastic mucosa covers the congested and hypercellular submucosa (X180, hematoxylin and eosin).

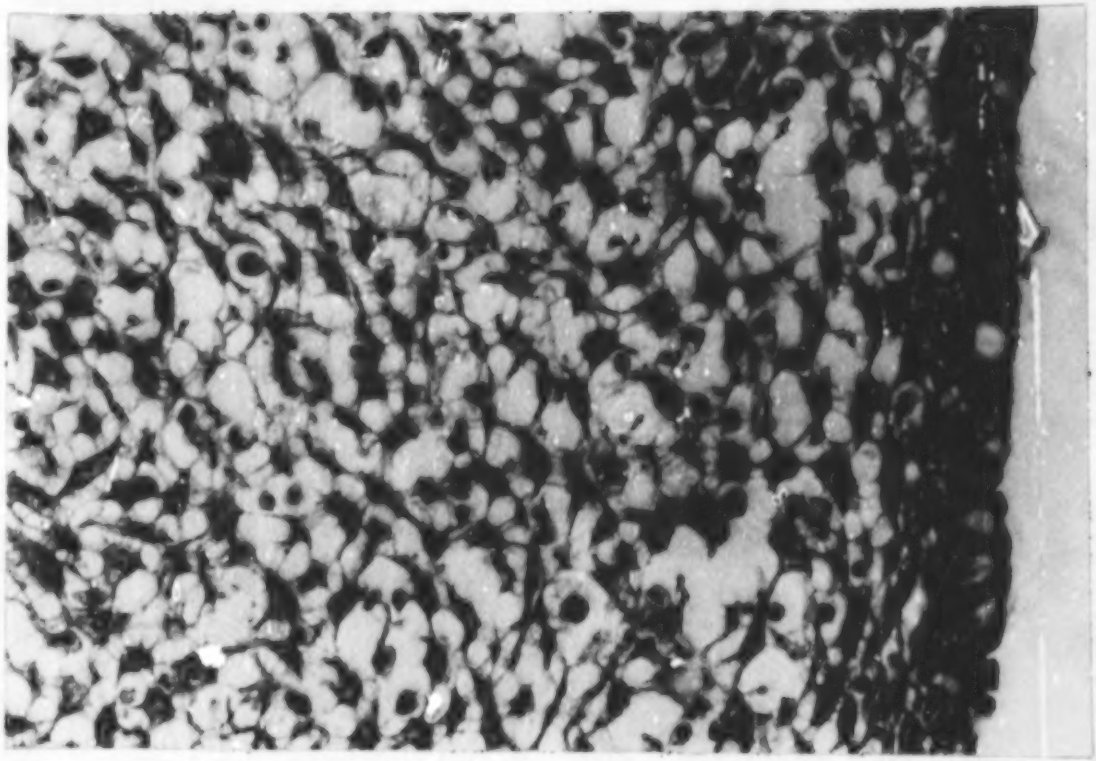

Figure 8. A superficial area of the lesion in Figure 7. Most of the mucosa consists of stellate cells. (X550, hematoxylin and eosin).

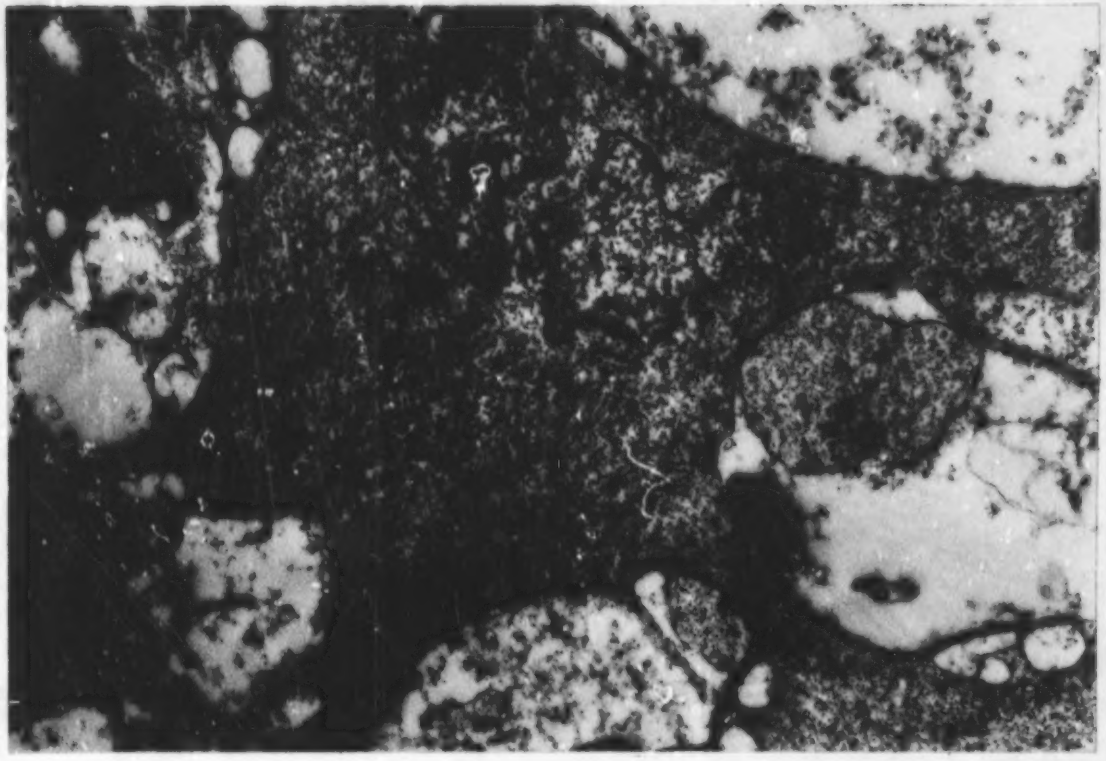

Figure 9. Electron micrographs of a black bullhead papilloma similar to the lesion in Figure 4. Stellate cells are separated by intercellular space containing amorphous material. Most of the cytoplasm consists of fibrils. (X17,000)

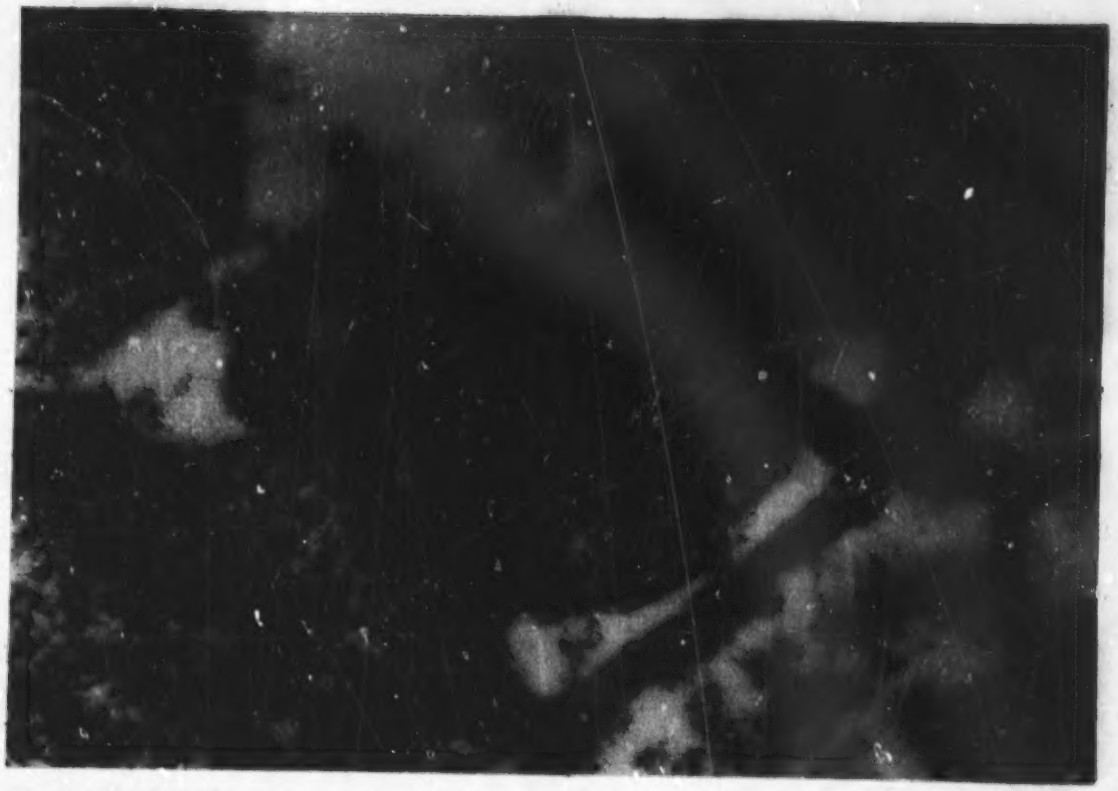

Figure 10. Enlargement of an area from Figure 9 showing a desmosome binding adjacent cells together (X50,000)

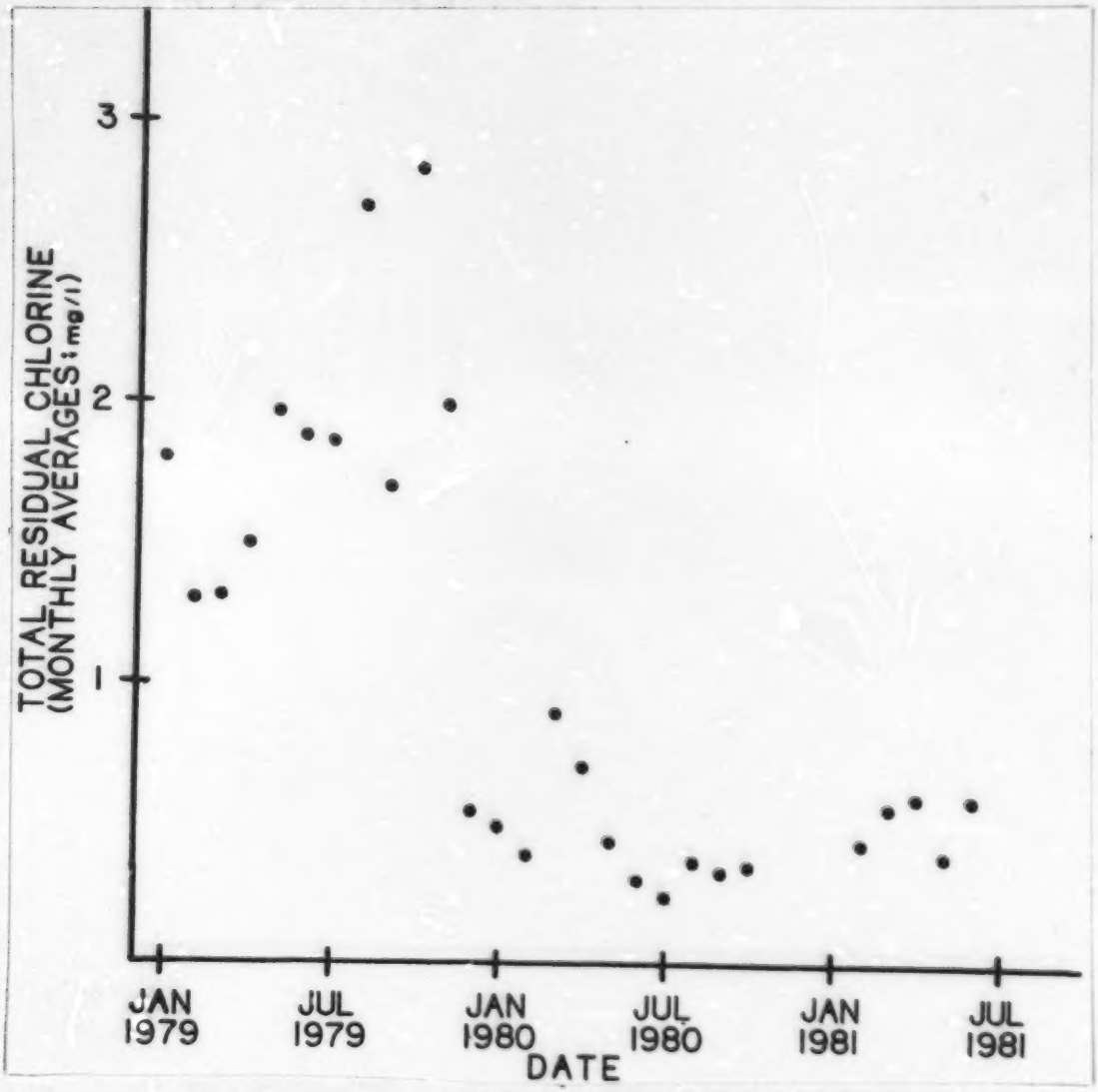

Figure 11. Total residual chlorine in water leaving the chlorine contact chamber of the South Tuskegee Water Pollution Control Plant.

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Metal concentrations in a sample collected from the oxidation pond on 1 November 1979 were zinc, 0.85 mg/1; copper, 0.04 mg/1; cadmium, 0.02 mg/1; manganese, 0.18 mg/1; and iron, 0.40 mg/1. Concentrations of these metals in the receiving stream above the outfall from the pond were higher than in the pond, except for zinc and copper, which were 0.42 mg/1 and 0.03 mg/1, respectively.

There were no signficant pesticide residues in water samples collected 16 November 1979 and 9 March 1981 or in soil samples collected 9 March 1981 from five locations in the oxidation pond.

Water samples collected on 1 May 1981 contained chloroform and bromodichloromethane (Figure 12) but chlorodibromomethane and bromoform were not found with a detection limit of 1.0 ug. Unidentified nitrogen and phosphorus organic residues were detected in the 100 ng/liter range.

MUTAGENICITY TESTS

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The acidic fraction of the oxidation-pond water collected at the pond outlet during June 1931 was mutagenic to Salmonella typhimurium strains TA98 and TA100 when S-9 was included in the agar but was not mutagenic if S-9 was omitted (Figures 13 and 14). The basic fraction was not mutagenic, with or without S-9, to Salmonella typhimurium strains TA98 and TA100.

TUMOR TRANSMISSION

No tumors or hyperplastic lesions occurred in black bullheads injected with homogenized papillomas or in controls. The adult fish were observed for 14 months and then necropsied. Juvenile fish have been observed for 140 days and will be observed for several more months.

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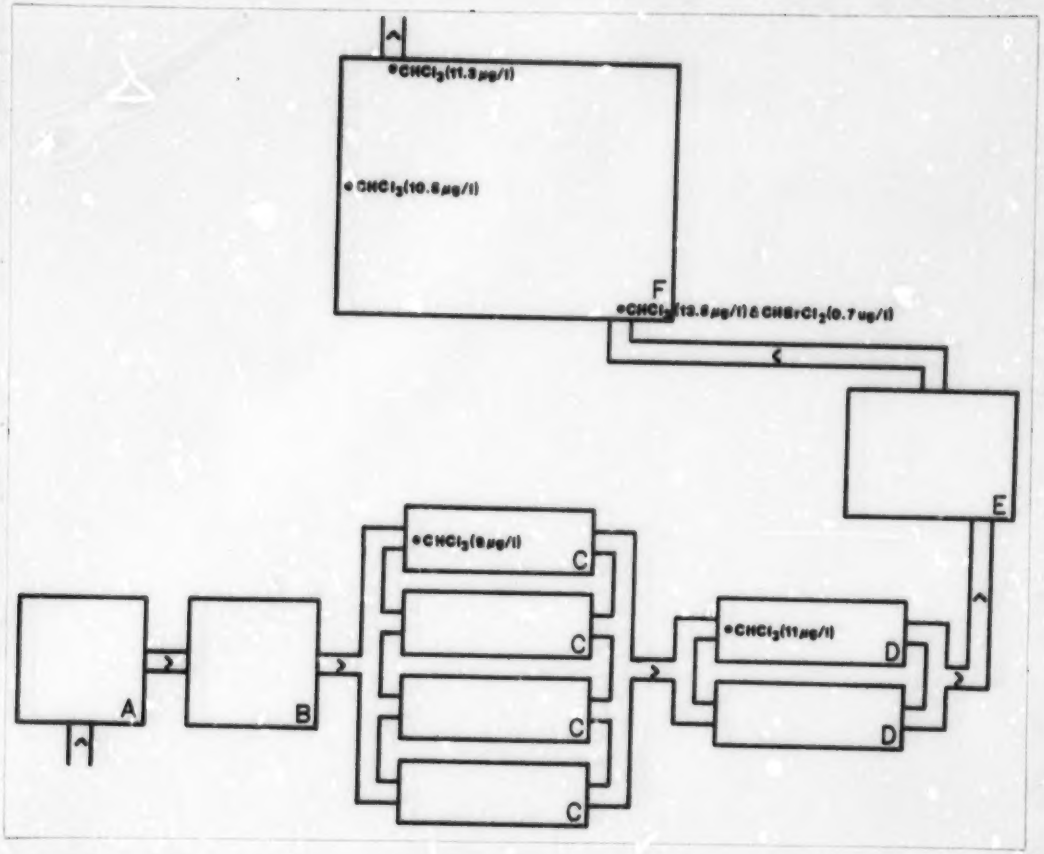

Figure 12. Concentrations of chloroform ($CHCl_3$) and bromodichloromethane ($CHBrCl_2$) from water samples collected on 1 May 1981. A, pumping station; B, grit removal; C, aeration basins; D, settling basins; E, chlorine contact chamber; F, final oxidation pond.

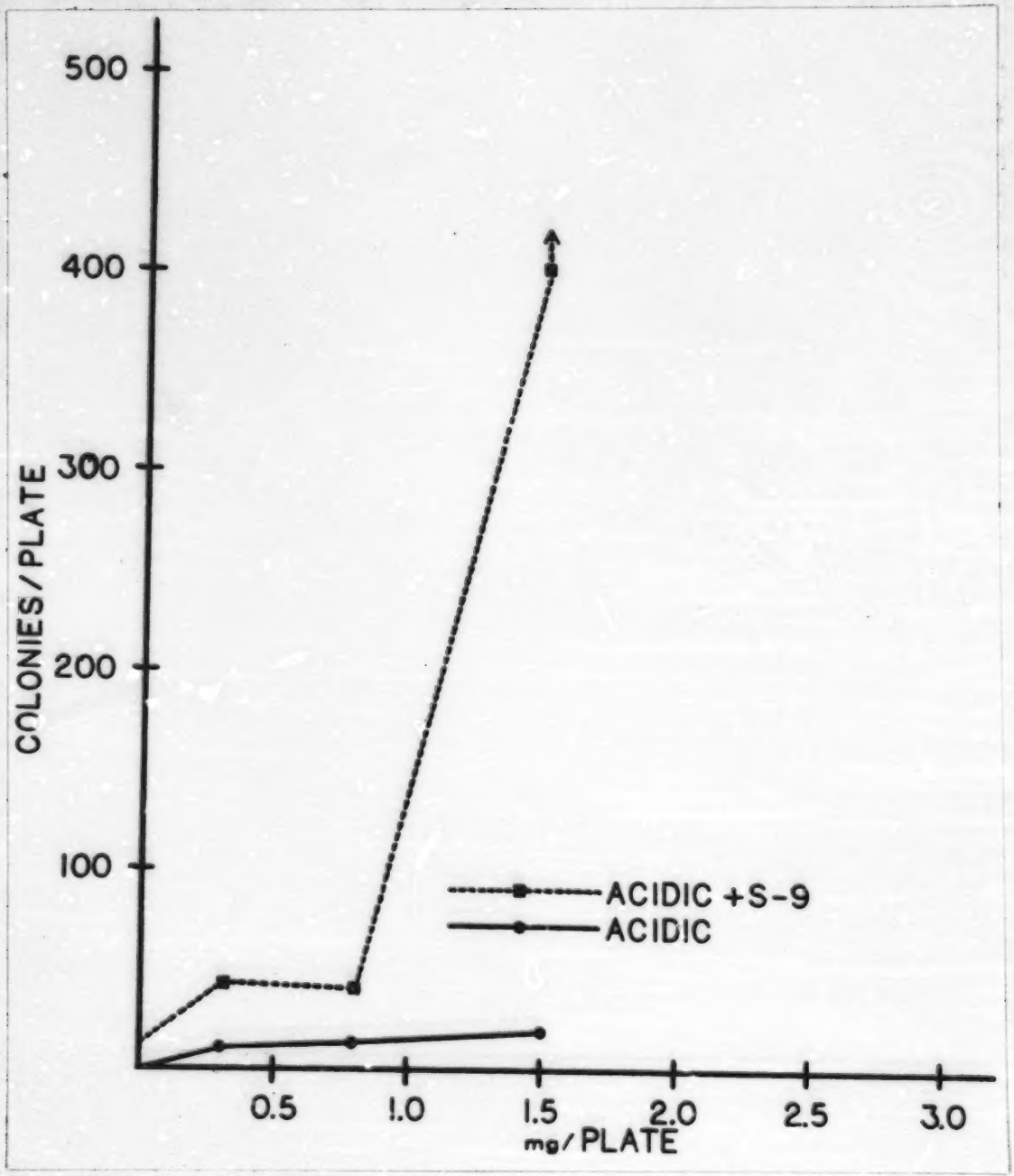

Figure 13. Response of Salmonella typhimurium strain TA98 in the Ames test to an acidic organic solvent extract of the final oxidation pond water.

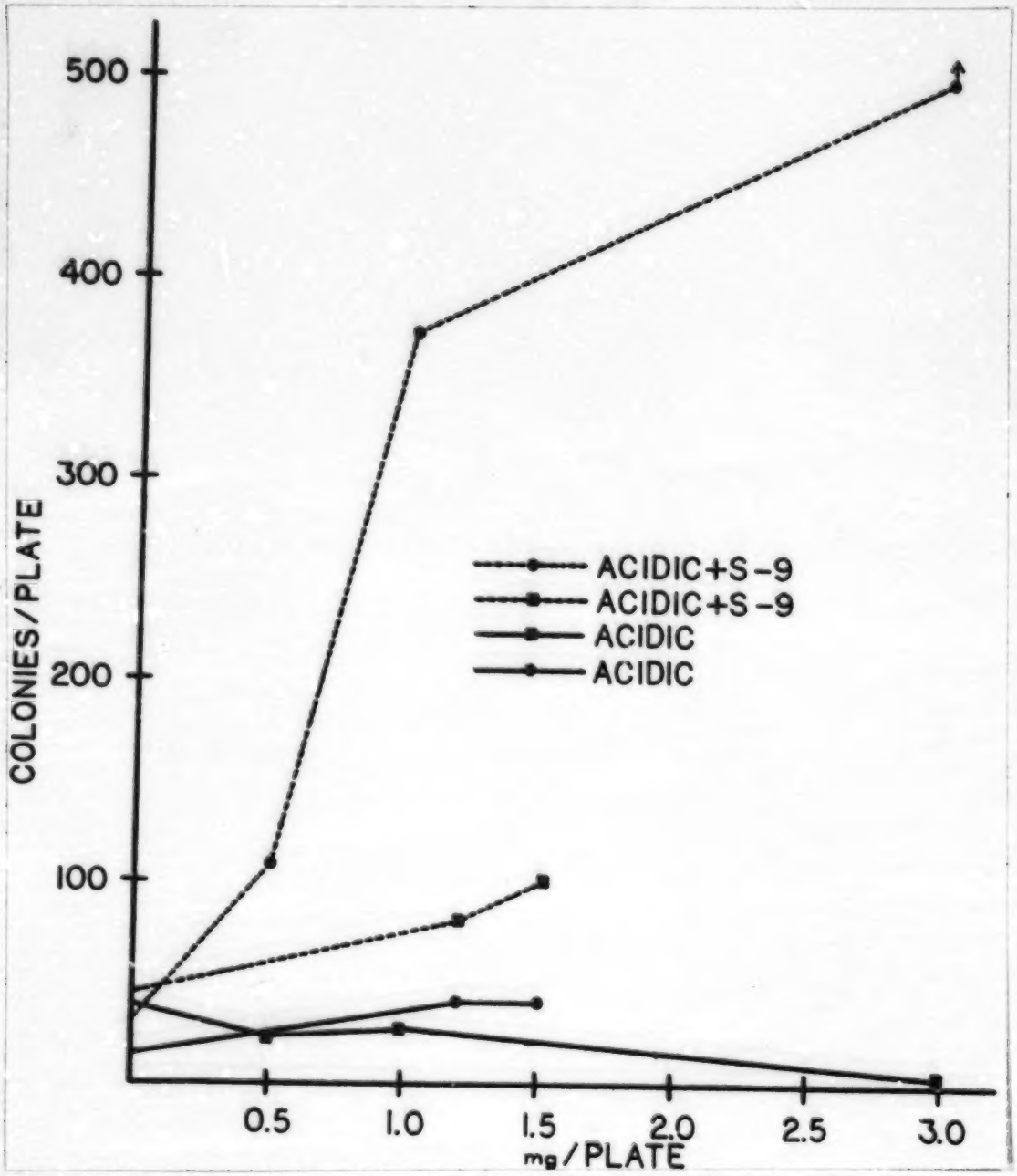

Figure 14. Response of Salmonella typhimurium strain TA100 in the Ames test to an acidic organic solvent extract of the final oxidation pond water. The results for two trials are given on this graph.

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SECTION 6

DISCUSSION

The papillomas described in this study resemble previously reported bullhead papillomas (Lucke and Schlumberger 1941; Steeves 1969). In eel papillomas, stellate epithelial cells and "dark cells" with 30-nm particles similar to those of the black bullhead papillomas were reported (Koops et al. 1970, Peters and Peters 1977). The neoplastic nature of oral papillomas in the black bullheads from the Tuskegee final oxidation pond was indicated by their persistence for up to a year after removal of the fish from the pond. The agent causing the papillomas did not cause neoplasia in green sunfish and golden shiners inhabiting the final oxidation pond.

Chemical carcinogens and viruses have been suggested as causes of fish neoplasms including papillomas (Mawdesley-Thomas 1975; Sonstegardt 1977). Evidence for chemicals causing papillomas has been the association between occurrence of fish tumors and polluted water (Dawe and Harshbarger 1975). The black bullhead population in the Tuskegee sewage treatment plant was similar to previously reported cases of fish in a polluted environment having a high prevalence of tumors while populations in nearby, unpolluted environments had no tumors.

The water analysis did not reveal substances suspected of causing tumors, but carcinogens could have been present during periods between sample collections. The absence of pesticides and other synthetic organic compounds in sediment samples indicated that any chemical carcinogens entering the pond were not accumulating in sediments.

A viral etiology seems likely for some fish papillomas. Several different types of viral particles were isolated from eels with stomatopapilloma; however, injection of the isolated eel viruses did not produce papillomas (see review by Pilcher and Fryer 1980). Viral particles seen in brown bullhead papillomas (Edwards et al. 1977) were 50-nm in diameter with a capsid. There is no evidence to date for a viral etiology for the tumors described in the present study, except for inclusion bodies in some epithelial cells of the papilloma. These inclusion bodies could be the result of degenerative changes in the cells and require further study for identification. The 35-nm particles seen in the black bullhead papillomas (Grizzle et al. 1981) are probably glycogen or other normal cell components rather than viruses. However, particles of the same size and general appearance occur in eel stomatopapilloma and are in electron-dense cells resembling those seen in black bullhead papillomas (Koops et al. 1970).

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Caged fish in the oxidation pond were exposed to both chemicals and pathogens that might have been present. Because the fish used in this experiment were of a different genetic stock, development of papillomas would have eliminated a genetic etiology. Tumors did not develop during the project period, but these fish will continue to be maintained in cages. Etiology of the oral mucosa hyperplasia and stomatitis occurring in caged fish was not determined, but the high incidence in fish in the oxidation pond suggested that chemicals or pathogens present in this pond were involved. Bacterial and parasitic pathogens were not associated with the lesions, but viruses could not be eliminated as a possible cause. Oral mucosa hyperplasia may occur spontaneously or be caused by an endemic pathogen because the lesions also occurred in control fish.

The reduced prevalence of oral mucosa lesions in caged black bullheads during the summer months could be a result of recovery from an infectious disease but could also result from acclimation to or decrease in concentration of a toxicant in the final oxidation pond. The high incidence of lesions in black bullheads confined to floating cages indicated that contact with the sediment or ingestion of benthic food organisms was not necessary for development of the hyperplastic lesions. Black bullheads were more susceptible to the agent causing the oral mucosa lesions than yellow bullheads and channel catfish, but effects of cage location on lesion development could not be determined because too few fish survived near the inlet. Water near the inlet was toxic to unacclimated fish, but the reason for this toxicity was not determined.

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